

In re Appln. of HISADA et al.
Application No. 09/622,206

SPECIFICATION AMENDMENTS

Please replace the paragraph beginning at page 3, line 25 with:

In recent years, in many cases, a minor constituent in a living body is analyzed by the electrophoresis mentioned above ~~in many cases~~. When performing such analysis, an immune complex formed by reacting a minor constituent in a living body with the antibody that recognizes the minor constituent as an antigen is detected. The immune complex is ~~preferably~~ preferably labeled with a fluorescent dye for the purpose of accurate detection. In this case, either the antigen or the antibody needs to be fluorescently labeled. Upon labeling the antigen or the antibody using the fluorescent dye, conventional labeling method can not be applied ~~due to~~ for the following reasons.

Please replace the paragraph beginning at page 4, line 11 with:

Antibodies and many of antigens are composed of proteins. The number of amino groups at an N-terminal of a protein and at a lysine side chain, and the dissociated state thereof are ~~a great factor for~~ factors in determining ~~an~~ the isoelectric point of a protein (Zokuseikagaku Jikkenkoza 2, Chemistry of proteins, the volume 1, Society of Japan Biochemistry, 1987). Therefore, the conventional labeling method utilizing a reaction between a fluorescent dye and an amino group of a protein greatly changes ~~an~~ the isoelectric point of a protein.

Please replace the paragraph beginning at page 5, line 6 with:

In addition, there is a ~~case that~~ situation where an analysis by the isoelectric focusing can not be done with high accuracy even when a monoclonal antibody having a uniform molecular weight obtained by a hybridoma is used as an antibody for detection. This is because the monoclonal antibody having a uniform molecular weight produced by hybridoma does not necessarily have a uniform isoelectric point, and this phenomenon is called microheterogeneity (Bouman H et al., Z Immunitatsforsch Exp Klin Immunol. 1975 Oct; 150 (4): 370-7).

Please replace paragraph beginning at page 5, line 16 with:

~~As a reason~~ reasons for this ~~ununiformity~~ non-uniformity of an isoelectric point, ~~there have been proposed~~ deamidation of a protein (Robinson A. B. et al., Proc. Natl. Acad. Sci. U.S.A. 1970 Jul; 66 (3): 753-7), pyroglutamylation of an N-terminal (Scott D.

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I. Et al., Biochem. J. 1972 Aug; 128(5): 1221-7), addition of a sugar chain (Cohenford M.A. et al. Immunol. Commun. 1983; 12(2): 189-200), myristoylation (Pillai S. et al., Proc. Natl. Acad. Sci. U.S.A. 1987 Nov; 84(21): 7654-8) and the like have been proposed, but the mechanism for the ~~ununiformity~~ non-uniformity of an the isoelectric point of a protein has not been specified yet.

Please replace paragraph beginning at page 6, line 1 with:

Therefore, when an analytical sample shows plural isoelectric points by isoelectric focusing, it is difficult to determine where the plurality ~~is originated~~ originates from, ~~because This is because~~ the plurality is ascribable either to an antigen or to an antibody. ~~This is because both~~ Both antigen and antibody may have ~~ununiformity~~ non-uniformity of an isoelectric point, as mentioned above.

Please replace paragraph beginning at page 6, line 15 with:

As Shimura K. and Karger B.L. disclose a method for quantitatively detecting an antigen using an antibody having a uniform isoelectric point, ~~a method of Shimura K. and Karger B.L. is known~~ (see Anal. Chem. 1994 Jan. 1; 66(1): 9-15, or JP-A 8-506182). The method disclosed in these references is schematically shown in Figs. 8A to G. That is, IgG antibody produced by a hybridoma (Fig. 8A) is cut with a protease (pepsin) and the resulting F(ab')₂ antibody (Fig. 8B) is separated. This is treated with a reducing agent of mercaptoethylamine to reduce three disulfide bonds (S-S bond) to obtain Fab' antibody (Fig. 8C). This Fab' antibody is oxidized to ~~make leave behind~~ only one reactive thiol group (SH group) left (Fig. 8D) and a fluorescent dye is bound to this thiol group (Fig. 8E). The resulting fluourescently labeled Fab' antibody is separated by the isoelectric focusing and a fluourescently labeled Fab' antibody having a uniform isoelectric point is taken from an ~~electrophoresis~~ electrophoresis carrier (Fig. 8F). The obtained fluourescently labeled Fab' antibody having a uniform isoelectric point is combined with an antigen. Then, electrophoresis is performed, and fluorescence caused by an excitation light is measured. (Fig. 8G).

Please replace paragraph beginning at page 7, line 12 with:

However, ~~when performing the isoelectire focusing by the method disclosed in the above mentioned references~~ by Shimura K and Karger BL, as above, involve steps for obtaining Fab' antibody having a uniform isoelectric point that are complicated. In addition,

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when an isoelectric point of the antigen, which is the analyte, is close to an isoelectric point of the fluorescently labeled antibody, migration time of the immune complex comprising antigen and antibody becomes almost the same as that of excessive antigen and/or antibody. Therefore, the electrophoretic peaks are overlapped overlap and detection can not be performed with high accuracy.

Please replace paragraph beginning at page 8, line 4 with:

The present inventors studied extensively and, as a result, found that it is possible to analyze an antigen with high accuracy by using an Fab' antibody having a uniform isoelectric point, which is has been modified by adding an amino acid sequence comprising a charged amino acid residue and by being labeled with a fluorescent dye, and which forms an immune complex with an antigen in an analytical sample.

Please replace paragraph beginning at page 17, line 9 with:

Fig. 7B is a view schematically showing the Fab' antibody having a uniform isoelectric point which was produced by antibody induction with IPTG.

Please replace paragraph beginning at page 19, line 8 with:

In the case of human IgG1 antibody, the antibody has the structure in which two polypeptide chains called L chain (light chain) and two polypeptide chains called H chain (heavy chain) makes a Y-shaped pair, as shown in ~~the schematic view of Fig. 1.~~ Fab' antibody is a fragment in which a hinge region or a part thereof is added to an Fab fragment. In the Fab' antibody, an Fd chain (H chain which is in an N-terminal side from a hinge region) consisting of a VH region and a CH1 region, and an L chain consisting of a VL region and a CL region are bonded by -S-S- bond.

Please replace paragraph beginning at page 56, line 23 with:

A hybridoma producing anti-human ~~alpha-1-antitrypsin~~ alpha-1-antitrypsin antibody was made using human ~~alpha-1-antitrypsin~~ alpha-1-antitrypsin (manufactured by Carbiochem-Noviochem) as an immunization antigen according to the following method:

Please replace paragraph beginning at page 57, line 1 with:

A BALB/c mouse was immunized four times with the above immunogen, spleen cells were taken, cell fusion was performed using a cultured mouse marrow cell (x63Ag8) and

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polyethylene glycol and cloning was performed. The binding activity of the immunogen with the antibody in the culture supernatant of the resultant clone was measured by the enzyme-antibody method. A clone that was considered to be positively reactive was further confirmed using the indirect fluorescent method. Then, nine kinds of hybridomas that produce the ~~alpha-1-anititrypsin~~ alpha-1-antitrypsin antibody were established. The antibodies produced by these hybridomas are the ones that bind to the human ~~alpha-1-anititrypsin~~ alpha-1-antitrypsin. For preparing the Fd gene and the L chain (κ chain) gene of the Fab' antibody having a uniform isoelectric point described below, these cells that produce anti-human ~~alpha-1-anititrypsin~~ alpha-1-antitrypsin antibody having the anti-~~alpha-1-anititrypsin~~ anti-alpha-1-antitrypsin activity were used.

Please replace paragraph beginning at page 57, line 20 with:

A gene expressing anti-human ~~alpha-1-anititrypsin~~ alpha-1-antitrypsin Fab' antibody was isolated from a hybridoma producing an IgG1 antibody against human ~~alpha-1-anititrypsin~~ alpha-1-antitrypsin as follows:

Please replace paragraph beginning at page 57, line 24 with:

That is, the ~~total~~ total RNA was extracted from the cells producing the anti-human ~~alpha-1-anititrypsin~~ alpha-1-antitrypsin Fab' antibody according to the protocol of BioMag mRNA purification kit (PerSeptive), and a single-stranded cDNA was synthesized using cDNA Synthesis System Plus (manufactured by Amersham Pharmacia Biotech Inc.). Polymerase chain reaction (PCR) was performed using the aforementioned cDNA as a template and using a DNA primer for isolating the Fd chain gene and a DNA primer for isolating the L chain gene, which were synthesized based on the base sequence of the variable region (V region) and the constant region (C region) classified by Kabat et al. (Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, NIH, Washington DC, 1991). Here, for designing a primer, reference was made to Hoogenboom H.R. et al. (Nucleic Acids Res., 1991, Aug 11:19 (15): 4133-7), and Kang A.S. et al. (Methods (San Diego) (1991), 2 (2), 111-18).

Please replace paragraph beginning at page 58, line 15 with:

In order to express an antibody which binds to the human ~~alpha-1-anititrypsin~~ alpha-1-antitrypsin as an Fab' antibody, a DNA primer was designed so that both the heavy chain (H chain) and the light chain (L chain) contain constant region. That is, a 5' primer (F5-1

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primer shown below) and a 3' primer (F3 primer shown below) were designed as a DNA primer for isolating the Fd chain gene, and a 5' primer (Kapper5 primer shown below) and a 3' primer (K3-1 primer shown below) were designed as a DNA primer for isolating the L chain gene.